

1 Proteomic analysis of pancreatic cancer stem cells: functional role of fatty acid synthesis and
2 mevalonate pathways

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24 **ABSTRACT**

25 Recently, we have shown that the secretome of pancreatic cancer stem cells (CSCs) is characterized by
26 proteins that participate in cancer differentiation, invasion, and metastasis. However, the differentially
27 expressed intracellular proteins that lead to the specific characteristics of pancreatic CSCs have not yet
28 been identified, and as a consequence the deranged metabolic pathways are yet to be elucidated.

29 To identify the modulated proteins of pancreatic CSCs, iTRAQ-based proteomic analysis was
30 performed to compare the proteome of Panc1 CSCs and Panc1 parental cells, identifying 230
31 modulated proteins. Pathway analysis revealed activation of glycolysis, the pentose phosphate
32 pathway, the pyruvate-malate cycle, and lipid metabolism as well as downregulation of the Krebs
33 cycle, the spliceosome and non-homologous end joining. These findings were supported by
34 metabolomics and immunoblotting analysis. It was also found that inhibition of fatty acid synthase by
35 cerulenin and of mevalonate pathways by atorvastatin have a greater anti-proliferative effect on cancer
36 stem cells than parental cells.

37 Taken together, these results clarify some important aspects of the metabolic network signature of
38 pancreatic cancer stem cells, shedding light on key and novel therapeutic targets and suggesting that
39 fatty acid synthesis and mevalonate pathways play a key role in ensuring their viability.

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43 **Introduction**

44 Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive solid tumours with a
45 mortality projected to surpass that of breast and colorectal cancer by 2030 in the United States [1].
46 More than 85% of patients who undergo surgical resection of small pancreatic tumours with clear
47 surgical margins and no evidence of metastasis, die from metastasis within 5 years [2, 3], a finding that
48 is consistent with early spread. Standard chemo- and radiation therapies, as well as new treatments
49 targeting known oncogenes or growth factors, do not offer significant improvement of survival [4]. In
50 line with these clinical observations, in a mouse model of PDAC, cellular dissemination leading to
51 liver metastasis has been demonstrated to occur prior to the formation of an identifiable primary
52 tumour. In addition, these circulating pancreatic cells have been shown to exhibit a mesenchymal
53 phenotype and the expression of typical markers of cancer stem cells (CSCs). The CSC theory of
54 cancer development is now generally accepted to explain the cellular heterogeneity observed within a
55 tumour. Following this paradigm, only small subpopulations of the tumour cells, the CSCs, are capable
56 to self renew, to give rise to a tumour and to recapitulate its heterogeneity by residing at the top of the
57 cellular hierarchy. CSCs potentially explain several phenomena of cancer such as minimal residual
58 disease, resistance to chemo- and radiation therapies, cancer recurrence and metastases [5]. Stem cells
59 from several cancers, both liquid and solid, including PDAC, have been identified and shown to be
60 particularly resistant to a broad spectrum of anticancer drugs [6]. The existence of CSCs has attractive
61 prospective for identification of CSC-targeted therapies through the determination of the crucial
62 molecules regulating the unique CSC properties. However, despite the enormous potential of CSCs as
63 a new diagnostic and therapeutic target for human cancers, the specific molecular features of these
64 cells are still far to be clarified mainly because of the difficulty to isolate sufficient amount of CSCs
65 from tissue samples. Recently, cancer cell lines have been shown to be an alternative source for CSC
66 research. Along these lines, our group has been able to isolate cancer stem-like cells from five out of
67 nine PDAC cell lines [7] and has demonstrated that Panc1 CSCs showed the highest tumoursphere-
68 forming ability and were the most resistant to the action of various anticancer drugs. In order to deepen

69 the knowledge of the specific molecular features of these cells, a proteomic approach has then been
70 chosen [8]. In particular, the secretome analysis of Panc1 CSCs has identified a total of 43 proteins
71 secreted at higher level by pancreatic CSCs compared to the parental cell line [9]. These data, together
72 with ELISA assays performed on sera of PDAC patients, has suggested that at least one of the highly
73 secreted proteins by CSCs, i.e. ceruloplasmin, is a promising marker for patients negative for CA19-9
74 [9].

75 Here, we report the proteomic analysis of the intracellular proteins of Panc1 CSCs and parental
76 cells. We show that in Panc1 CSCs 115 proteins were up-regulated and 115 down-regulated as
77 compared to parental cells. *In silico* functional pathway analysis and network reconstruction based on
78 signalling reactions reported in literature demonstrates a predominant association of the up-regulated
79 proteins with glycolysis/gluconeogenesis, pentose phosphate pathway (PPP), pyruvate-malate cycle,
80 and lipid metabolism and of down-regulated proteins with Krebs cycle, spliceosome and non-
81 homologous end joining. A metabolomic analysis on glycolysis, Krebs cycle and PPP confirmed the
82 modulation of these pathways. Among the identified proteins, fatty acid synthase (FASN) and
83 acetoacetyl-CoA transferase (ACAT2) were among the most highly up-regulated and were chosen for
84 further analysis. Our data indicate that treatment of cells with cerulenin, a specific FASN inhibitor, or
85 with atorvastatin, a specific inhibitor of the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
86 (HMGCR), an enzyme located downstream to ACAT2 in the metabolic pathway that produces
87 isoprenoids and cholesterol, preferentially decrease cell viability of Panc1 CSCs compared to parental
88 cells. All our findings constitute a significant advance in the comprehension of PDAC CSC biology
89 and provide interesting potential targets for the therapeutic approaches to PDAC designed to
90 specifically eliminate the CSC cellular component of the tumour.

91

92 MATERIALS AND METHODS

93 Cell culture

94 The human PDAC cell line Panc1, called here Panc1 parental cells, was grown in RPMI 1640
95 supplemented with 10% FBS, 2 mM glutamine, and 50 µg/ml gentamicin sulfate (Gibco, Life
96 Technologies). Adherent cells were maintained in standard conditions for a few passages at 37°C with
97 5% CO₂. Panc1 CSCs were obtained as previously described [7]. Briefly, adherent cells were cultured
98 in CSC medium (i.e. DMEM/F-12, B27, fungizone, penicillin/streptomycin, heparin, epidermal
99 growth factor and fibroblast growth factor) for at least 1-3 weeks or until the appearance of
100 tumourspheres, which were then cultured in CSC medium for at least three passages before initiating
101 the experiments.

102

103 Sample preparation

104 Samples were prepared as previously described [9]. Briefly, Panc1 cells and Panc1 CSCs cell
105 pellet was collected, lysed in 0.5 M TEAB (Sigma) and 0.1% SDS supplemented with protease
106 inhibitor cocktail 1X (Roche), sonicated 3 times for 10 sec, stored at -80°C for 30 minutes and then
107 sonicated again 3 times for 10 sec. Samples were then centrifuged at 14,000 x g for 10 min at 4°C to
108 remove debris, and the supernatants were collected and stored and -80°C. Protein concentration was
109 determined using BCA protein assay (Thermo Scientific).

110

111 Protein digestion and iTRAQ labeling

112 For this experiment, iTRAQ 8-plex reagents (Sciex, Framingham, USA) were used for the
113 simultaneous analysis of the conditioned media [9] and whole cell lysates of Panc1 cells and Panc1
114 CSCs. The samples labeled with 114, 116, 117, and 121 iTRAQ tags were used for the secretome
115 analysis of and the data have already been published. In the present work were instead analyzed the
116 samples labeled with 113, 115, 117, and 119 tags. In particular, one biological replica of Panc1 cell
117 line and of Panc1 CSC whole cell lysates were labeled with iTRAQ reagent 113 and 115, respectively,

118 and a second biological replicate (from a different cell culture passage) was labeled in the same order
119 with iTRAQ reagents 117 and 119. Protein digestion, iTRAQ labeling, and peptide fractionation and
120 desalting were carried out as already described [9].

121

122 **LC-MS/MS analysis and data processing**

123 Desalted fractions were reconstituted in 40 μ L 0.1% formic acid and 5 μ L aliquots were
124 delivered into a Triple TOF 5600 (Sciex) via an Eksigent NanoUltra cHiPLC System (Sciex) mounted
125 with a microfluidic trap (200 μ m x 500 μ m ChromXP C18-CL 3 μ m 300 Å) and analytical column (15
126 cm x 75 μ m) packed with ChromXP C₁₈-CL 3 μ m. A NanoSpray III source was fitted with a 10 μ m
127 inner diameter SilicaTip emitter (New Objective, Woburn, USA). The trap column was washed with
128 2% ACN/0.1% formic acid for 10 min at 2 μ L/min. A gradient of 2–50% ACN/0.1% formic acid (v/v)
129 over 90 min was applied at a flow rate of 300 nL/min. Spectra were acquired automatically in positive
130 ion mode using information-dependent acquisition powered by Analyst TF 1.5.1 software (Sciex). Up
131 to 25 MS/MS spectra were acquired per cycle (approximately 10 Hz) using a threshold of 100 counts
132 per s and with dynamic exclusion for 12 s. The rolling collision energy was increased automatically by
133 selecting the iTRAQ check box in Analyst, and manually by increasing the collision energy intercepts
134 by 5. TOF-MS spectra were acquired for 250 ms (mass range 400–1650 Da) and MS/MS spectra for
135 100 ms each (mass range 100–1400 Da). Mass spectrometer recalibration was performed at the start of
136 every fifth sample using a β -galactosidase digest standard.

137 Data analysis was performed using ProteinPilot software (Version 4.2, revision 1340, Sciex)
138 using default settings and with bias and background correction applied. The data were searched against
139 UniProt/SwissProt database (2013_2, total 30,309,316 entries, 40,464 human entries searched) using
140 the Paragon algorithm (4.2.0.0, version 1304, Sciex). The mass tolerance for both precursor and
141 fragment ions was 10ppm [10]. The variable modifications selected for the search were ‘biological
142 modifications’ (probability-based modification search of 461 biological, chemical and artefactual
143 modifications), while the fixed modifications were carbamidomethylation of cysteines, and iTRAQ

144 modification of C-terminal lysine residues and peptide N-termini. A global FDR value of 1% was used
145 based on the number of proteins identified before 1% of the identifications were derived from a match
146 to the reverse database [11] (equating to an unused score of 1.09 and a confidence of 91.9%).
147 Similarly, a global FDR cut-off of 1% was used as the criterion for acceptance of individual MS/MS
148 spectra and in this case corresponded to a confidence of 93.8%.

149 Ratios were calculated from the areas under the curve for each iTRAQ reporter ion selecting
150 different denominators depending on the comparisons to be made. Mean ratios were calculated based
151 on all occurrences (up to 7) of all peptides for which there was a peptide confidence of >15% and
152 where the protein was confidently identified through other evidence. Where a single peptide was used
153 for quantification, the peptide confidence cut-off was 95%. Where a single peptide was used for
154 identification, the cut-off was 99%. The Paragon algorithm performs a Student t-test on the
155 unweighted log ratios (for background corrected data) and reports the p-value: for a final error rate of
156 5% and with 1157 proteins quantified, the Bonferroni correction suggests a significant p-value at
157 0.0043.

158

159 **Bioinformatics analysis of identified proteins**

160 Known and predicted protein associations were analyzed and visualized with STRING version
161 10 software (<http://stringdb.org/>). We retrieved interactions that were of at least high confidence (score
162 0.7), based exclusively on experimental and database knowledge, while excluding all other prediction
163 methods implemented in STRING (such as text-mining and co-expression). Additional white nodes
164 and network depth were kept to the minimum value (1), in order to exclude as many false positive
165 interactions as possible.

166 Moreover, Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA) was used
167 to perform a comprehensive analysis of modulated proteins that characterized Panc1 CSCs. The IPA
168 Core Analysis allowed to identifying the most significant networks, biological functions, perturbed
169 canonical pathways as well as potential upstream regulators associated with this signature. The settings

170 were as follows: i) Reference set: Ingenuity Knowledge Base; ii) Relationship to include: Direct and
171 Indirect; iii) Filter Summary: Consider only molecules and/or relationships where (species = Human)
172 AND (confidence = Experimentally Observed).

173 The most important networks were calculated on the basis of the IPA score (>40) which take
174 into account the number of focus proteins and the size of the network to approximate the relevance of
175 the network to the original list of proteins. Proteins associated with canonical pathways were estimated
176 as significant using Fisher's exact test ($p\text{-value} \leq 0.01$) to determine the probability that the association
177 between identified proteins and a canonical pathway could be explained by chance alone.

178 We also performed the IPA Upstream Regulator analysis to identify a putative cascade of
179 upstream transcriptional regulators that can further explain the observed expression changes in Panc1
180 CSCs. The upstream regulators were assumed as valid effectors of gene/protein expression if the
181 corresponding p-value obtained by Fisher's exact test was ≤ 0.01 . Activation z-score algorithm was
182 used to allow for prediction whether an upstream regulator is activated ($z \geq 2$) or inactivated ($z \leq -2$)
183 based on the direction of expressional change of the associated genes.

184

185 **Western Blot analysis**

186 Western Blot analysis was performed on two independent biological replicates of Panc1 and
187 Panc1 CSCs to validate quantitative data obtained by MS, and on a biological replicate of Panc1 CSCs
188 to verify the level of expression of FASN and of ACAT2 after inhibition of fatty acid synthesis and
189 mevalonate pathways.

190 Protein samples were diluted 1:1 with Laemmli's sample buffer (62.5 mM Tris-HCl, pH 6.8,
191 25% glycerol, 2% SDS, 0.01% Bromophenol Blue), heated for 5 min at 95°C and separated by
192 SDS/polyacrylamide gel electrophoresis (PAGE) on 12% T acrylamide gels in Tris/glycine/SDS
193 buffer. Proteins were then electroblotted onto polyvinylidene fluoride membranes (Bio-Rad, Hercules,
194 CA) at 80 V for 1h and 30 min at 4°C. Amido Black staining was used to confirm equal protein
195 loading in different lanes. Non-specific sites were blocked by incubating the membranes with 5% non-

fat dried milk and 0.05% Tween-20 (Sigma-Adrich) in Tris-buffered saline for 1h at room temperature. Membranes were incubated with the primary antibodies at the appropriate dilutions in 1% non-fat dried milk, 0.05% Tween-20 in Tris-buffered saline for 3 h at room temperature. Blots were then incubated 1h at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (see the **Supplemental Table 1**). The immunocomplexes were visualized by chemiluminescence using the Chemidoc MP imaging system (Bio-Rad Laboratories) and the intensity of the chemiluminescence response was measured by processing the image with Image Lab software (Bio-Rad).

204

205 **Metabolite extraction**

Metabolomic analyses on Panc1 cells and Panc1 CSCs were performed as previously reported [12]. Cells were prepared following the protocol published by Sana et al. [13], with minor modifications as previously reported [12]. The sample was resuspended by adding 0.15 ml of ice-cold ultra-pure water (18MΩ) to lyse cells. The tubes were plunged into dry ice or a circulating bath at -25°C for 0.5 min and then into a water bath at 37°C for 0.5 min. To each tube was added 0.6 ml of -20°C methanol and then 0.45 ml of -20°C chloroform. The tubes were mixed every 5 min for 30 min. Subsequently, 0.15 ml of ice-cold pH-adjusted ultra-pure water was added to each tube and these were centrifuged at 1000xg for 1 min at 4°C, before being transferred to -20°C for 2–8 h. After thawing, liquid phases were recovered and an equivalent volume of acetonitrile was added to precipitate any residual protein. The tubes were then transferred to a refrigerator (4°C) for 20 min, centrifuged at 10,000xg for 10 min at 4°C and the collected supernatants were dried to obtain visible pellets. Finally, the dried samples were re-suspended in 1 ml of water, 5% formic acid and transferred to glass autosampler vials for LC/MS analysis.

219

220 **Rapid-resolution reverse-phase HPLC for metabolite separation**

221 An Ultimate 3000 Rapid Resolution HPLC system (DIONEX, Sunnyvale, USA) was used to perform
222 metabolite separation. The system featured a binary pump and vacuum degasser, well-plate
223 autosampler with a six-port micro-switching valve, and a thermostated column compartment. A
224 Phenomenex Luna 3 μ m HILIC 200A (150 \times 2.0 mm), protected by a guard column HILIC 4 \times 2.0 mm
225 ID (Phenomenex), was used to perform metabolite separation over a phase B to phase A gradient
226 lasting 35 minutes. For the HILIC separation, a solution of 50 mM ammonium acetate was prepared by
227 dissolving ammonium acetate in deionized water. Aqueous ammonium acetate was mixed with
228 acetonitrile (95:5, v/v). This was used for the mobile phase 'A'. The eluent 'B' was composed of a
229 mixture of 50 mM aqueous ammonium acetate: water plus acetonitrile (95:5), v/v). Acetonitrile,
230 formic acid, and HPLC-grade water and metabolite standards (\geq 98% chemical purity) were purchased
231 from Sigma Aldrich.

232

233 **Mass spectrometry: Q-TOF settings**

234 Due to the use of linear ion counting for direct comparisons against naturally expected isotopic
235 ratios, time-of-flight instruments are most often, being the best choice for molecular formula
236 determination. Thus mass spectrometry analysis was carried out on an electrospray hybrid quadrupole
237 time-of-flight instrument MicroTOF-Q (Bruker-Daltonik, Bremen, Germany) equipped with an ESI-
238 ion source. Mass spectra for metabolite-extracted samples were acquired in negative ion modes. ESI
239 capillary voltage was set at 4500 V (–) ion mode. The liquid nebulizer was set at 27 psi and the
240 nitrogen drying gas was set to a flow rate of 6 L/min. Dry gas temperature was maintained at 200 °C.
241 Data were stored in a centroid mode. Data were acquired with a stored mass range of 50–1200 m/z.
242 Because calibration of the mass analyzer is essential in order to maintain a high level of mass
243 accuracy, instrument calibration was performed externally every day with a sodium formate solution
244 consisting of 10 mM sodium hydroxide in 50% isopropanol: water, 0.1% formic acid. Automated
245 internal mass scale calibration was performed through direct automated injection of the calibration
246 solution at the beginning and at the end of each run by a 6-port divert-valve.

247 **Metabolite data elaboration**

248 Replicates were exported as mzXML files and processed through MAVEN.52; mass
249 spectrometry chromatograms were elaborated for peak alignment, matching and comparison of parent
250 and fragment ions, and tentative metabolite identification (within a 10 ppm mass-deviation range
251 between observed and expected results against the imported KEGG database). MAVEN is an open-
252 source software that could be freely downloaded from the official project websites ([http://genomics-](http://genomics-pubs.princeton.edu/mzroll/index.php?show=download)
253 [pubs.princeton.edu/mzroll/index.php?show=download](http://genomics-pubs.princeton.edu/mzroll/index.php?show=download)). Results were graphed with Graphpad Prism
254 5.01 (Graphpad Software Inc). Statistical analyses were performed with the same software, as a result
255 of paired t-test or two-way ANOVA among the results obtained from Panc1 and Panc1 CSCs.

256

257 **Cerulenin and atorvastatin treatments, cell proliferation assay and morphologic changes**

258 Cerulenin and atorvastatin were obtained from Sigma-Aldrich St. Louis, MO. Cerulenin was
259 dissolved in ethanol at a final concentration of 20 mg/ml and stored at -20°C. Atorvastatin was
260 dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) at a final concentration of 10 mg/ml and
261 stored at room temperature. Panc1 and Panc1 CSCs (5×10^3 cell/well) were cultured in 96-well plates
262 and incubated at 37°C with 5% CO₂. Twenty-four hours later, cells were treated with a serial
263 concentration of cerulenin (0, 5, 10, 25, 50, 100, 250, and 500 µM) or of atorvastatin (0, 2.5, 5, 10, 25,
264 50, 100, and 250 µM). Three independent experiments were performed.

265 After 48 h of each treatment, resazurin dye solution was added in an amount equal to 10% of
266 the culture medium volume and plates were incubated for 1 h at 37°C with 5% CO₂. Metabolic activity
267 of living cells was measured fluorometrically by monitoring the increase in fluorescence at a
268 wavelength of 590 nm, using an excitation wavelength of 535 nm on an automatic microplate reader.
269 The effect of inhibitors on cellular morphology was assessed by collecting phase-contrast microscopy
270 images of Panc1 and Panc1 CSCs cells after 48 h.

271

272 **Statistical analysis**

273 The data for inhibitors-treated cells and non-treated controls were compared using two-way
274 analysis of variance (ANOVA) and Student's t-test. P-values less than 0.05 were regarded statistically
275 significant. All data were processed using GraphPad Prism Software Version 6.0 (La Jolla, CA, USA).

276

277

278 **RESULTS**

279 **Differential profile of the whole cell proteome in Panc1 CSCs respect to Panc1 cells**

280 Protein expression profiles of Panc1 cells and Panc1 CSCs were investigated using the iTRAQ
281 approach. A total of 2045 proteins with at least 93.8% confidence and an Unused ProteinPilot scores >
282 1.09 (equating to a global FDR of 1%) were identified and among them 1157 proteins were quantified
283 (**Supplemental Table 2**). Of these, 608 were identified via a single peptide with a confidence of 99%
284 (**Supplemental Table 3**). Differential protein expression was considered to be significant when the
285 expression increased or decreased with a fold change of 1.5 and a p value smaller than 0.05 in both
286 biological replicates. A total of 230 proteins were found differentially expressed (**Supplemental Table**
287 **4**) and among them 115 proteins were up-regulated and 115 down-regulated in Panc1 CSCs as
288 compared to Panc1 parental cells.

289

290 **Interaction networks, pathway analyses, and upstream regulators of Panc1 CSC proteins**

291 STRING analysis emphasized that the majority of the differentially expressed Panc1 CSC
292 proteins interact within established complexes or have functional relationships.

293 The most striking pathways involving the proteins up-regulated in Panc1 CSCs compared to
294 parental cells belong to carbon metabolism and are, in particular, glycolysis/gluconeogenesis, pyruvate
295 metabolism, biosynthesis of amino acids, and pentose phosphate pathway (**Fig. 1**). On the contrary, the
296 down-regulated proteins are mainly involved in the interactions with molecular components of the
297 spliceosome and non-homologous end joining (**Fig. 2**).

298 To reveal significant networks and biological functions relevant to Panc1 CSCs, we also
299 performed an IPA. The up-regulated proteins show the highest-score for networks including cell death
300 and survival, cellular assembly and organization, cell-to-cell signalling and interaction, cellular
301 development, growth and proliferation, and carbohydrate metabolism, while the down-regulated
302 proteins are involved in cell death and survival, cellular growth and proliferation, and RNA post-
303 transcriptional modification (**Supplemental Fig 1**). Next, we systematically evaluated the biological
304 functions of the identified proteins. Most of the regulated Panc1 CSC proteins appear to be involved in
305 cancer, cellular growth and proliferation (**Table 1**). In particular, the up-regulated proteins are
306 specifically involved in cellular movement and free radical scavenging, while the down-regulated
307 proteins are specifically connected to the post-transcriptional modification of RNA and to cellular
308 response to therapeutics. Overall, IPA software revealed that the Panc1 CSC modulated proteins show
309 a significant link with 21 or 19 different pathways for up- or down-regulated proteins, respectively
310 (**Supplemental Table 5**). The ten top canonical pathways ($p\text{-value} \leq 0.001$) are shown in **Figure 3**.
311 Among the most statistically significant canonical pathways, glycolysis ($P\text{-value: } 7.94 \text{ E-}11$) and
312 gluconeogenesis ($P\text{-value: } 1.12 \text{ E-}10$) are included for the up-regulated proteins and DNA double-
313 strand break repair by non-homologous end joining ($P\text{-value: } 1.23 \text{ E-}06$) and telomere extension by
314 telomerase ($P\text{-value: } 1.66 \text{ E-}06$) for the down-regulated proteins.

315 Finally, the IPA software allowed us to examine also the potential upstream regulators
316 associated with the above-described proteomic profiles. In particular, the upstream regulators predicted
317 to be significantly activated include the transcription factors HIF1A ($p = 1.29\text{E-}09$, $z = 2.228$) and
318 SMARCA4 ($p = 5.36\text{E-}06$, $z = 2.219$), as well as the estrogen-related receptor gamma ESRRG ($p =$
319 $4.24\text{E-}07$, $z = 2.180$), while the transcription factor NR1H4 ($p = 2.08\text{E-}05$, $z = -2.236$) is predicted to
320 be inhibited in Panc1 CSCs. A complete list of transcriptional upstream regulators with significant p -
321 values ($p \leq 0.01$) can be found in **Supplemental Table 6**.

322

323

324 **Western blot analyses of selected proteins identified by iTRAQ**

325 Seventeen differentially expressed proteins (ten up-regulated and seven down-regulated in
326 Panc1 CSCs), for which commercial antibodies were available, were selected for validation using
327 Western blot analyses. The expression level of proteins in Panc1 cells and Panc1 CSCs were compared
328 on two biological replicates. As shown in **Figure 4**, Western blot results are consistent with the MS
329 quantification data. Notably, in Panc1 CSCs relative to parental cells, MARCKS is up-regulated at
330 intracellular level only as intact (~ 80 kDa), but not as cleaved (~ 40 kDa) form and more isoforms of
331 Integrin beta-1 (ITGB1) and hnRNP A2/B1 (HNRNPA2B1) are immunodetected and all appear to be
332 down-regulated.

333

334 **Metabolomic analysis of Panc1 CSCs**

335 Since the proteomic analysis revealed that carbon metabolism (mainly glycolysis and pyruvate
336 metabolism) is the pathway that characterizes the proteins induced in Panc1 CSCs (**Fig. 1**), we
337 determined fold-change variations of the concentration levels of several key metabolites of glycolysis,
338 Krebs cycle, and the energetic metabolism. **Figure 5A** shows that the glycolytic intermediates are
339 mainly present at higher level in Panc1 CSCs in comparison to Panc1 parental cells. In particular,
340 Panc1 CSCs show a significant increase in glucose-6 phosphate, fructose-1,6 biphosphate,
341 glyceraldehyde-3 phosphate, and lactate, while pyruvate decreases. We next investigated whether the
342 accumulation of glycolytic intermediates was associated to an increase in refueling of pentose
343 phosphate pathway (PPP). Interestingly, Panc1 CSCs reveal the accumulation of PPP intermediates,
344 both of the oxidative phase (D-gluconic acid and D-ribose 5-phosphate) and non-oxidative phase
345 (sedoheptulose 7-phosphate and D-xylulose phosphate) (**Figure 5B**). In line with the increased level of
346 glycolysis metabolites, Krebs cycle intermediates including succinate, fumarate, and malate are
347 decreased and two Krebs cycle-related metabolites, glutamine and glutamate, are decreased and
348 increased, respectively, in Panc1 CSCs compared to Panc1 P cells (**Figure 5C**).

349

350 **Decreased cell viability of Panc1 CSCs in response to inhibition of fatty acid synthesis and** 351 **mevalonate pathways**

352 The observation that FASN was expressed at 18 fold higher level in Panc1 CSCs compared to
353 Panc1 cells led us to investigate the role of fatty acid synthesis in Panc1 CSC viability. For this
354 purpose, we tested the effect of cerulenin, a specific FASN inhibitor, on the proliferative activity of
355 Panc1 cells and Panc1 CSCs. The cell viability assay was performed 24 (data not shown) or 48 hours
356 after the beginning of the treatment with cerulenin at concentrations ranging from 0 to 500 μ M. As
357 shown in **Figure 6A**, cerulenin is able to decrease cell viability at a significantly higher level in Panc1
358 CSCs compared to Panc1 cells with IC_{50} values of $15.6 \mu M \pm 1.3$ and $24.2 \mu M \pm 1.7$, respectively.
359 This result strongly suggests an increased cell viability role of fatty acid synthesis in CSCs compared
360 to parental cells.

361 In a parallel experiment, conceived on the observation that ACAT2 was expressed at 21 fold
362 higher level in Panc1 CSCs compared to Panc1 cells, we examined the role of isoprenoids/cholesterol
363 synthesis in Panc1 CSC viability. For this purpose, we used the drug atorvastatin, which is known to
364 specifically inhibit HMG-CoA reductase, an enzyme located downstream to ACAT2 in the metabolic
365 pathway that produces isoprenoids and cholesterol. We cultured both Panc1 cells and Panc1 CSCs for
366 24 (data not shown) and for 48 hour with atorvastatin at concentrations ranging from 0 to 250 μ M. As
367 shown in **Figure 6B**, atorvastatin strongly inhibits the viability of Panc1 CSCs, while only slightly
368 reduces that of Panc1 cells, with IC_{50} values of $43 \mu M \pm 24.8$ and $> 250 \mu M$, respectively. This result
369 strongly suggests an increased cell viability role of isoprenoids/cholesterol synthesis in CSCs
370 compared to parental cells.

371 To verify whether treatments with cerulenin and atorvastatin had an effect on the expression
372 level of FASN and ACAT2 and to ascertain that the up-regulation of FASN observed in Panc1 CSCs
373 was not a consequence of the presence of growth factors in the culture medium [14], a western blot
374 analysis was performed. As shown in **Figure 7**, FASN level is independent of the presence of EGF and
375 FGF in the culture medium and is not modulated by the treatment with the specific inhibitor cerulenin,

376 which is a covalent inactivator of the β -ketoacyl synthase reaction on FAS. On the contrary, the
377 expression level of ACAT2 in Panc1 CSCs appears to be increased after the inhibition of the
378 mevalonate pathway by atorvastatin.

379

380 **Morphologic changes of Panc1 cells and Panc1 CSCs in response to the inhibition of fatty acid** 381 **synthesis and mevalonate pathways**

382 To evaluate if the inhibition of fatty acid synthesis had an effect on cellular morphology, we examined
383 cells by a phase-contrast microscope at 48 hours after treatment with 0, 25, 100 or 250 μ M cerulenin.
384 Before treatment, Panc1 cells exhibited a typical epithelial morphology with intact cell-to-cell
385 contacts, while Panc1 CSCs exhibited a mesenchymal morphology with cell aggregates (or spheroids)
386 and a more dispersed colony appearance suggesting an epithelial mesenchymal transition (EMT)
387 phenotype. Interestingly, after cerulenin treatment, Panc1 cells exhibited only a decreased cell-to-cell
388 contact, while Panc1 CSCs showed drastic changes in cell morphology, with reduction of spheroids
389 suggesting cytoskeletal reorganization (**Fig. 8A**). The analysis also indicated that the anti-
390 proliferative/viability effect was due, at least in part, to the decrease in cell number after cerulenin
391 treatment.

392 We also evaluated the effect of atorvastatin on the morphology of Panc1 cells and Panc1 CSCs.
393 As shown in **Figure 8B**, the effect on cell morphology of this inhibitor was similar to that of cerulenin.
394 Phase-contrast microscopy images show in fact that after atorvastatin treatment Panc1 CSCs were
395 characterized by a reduction of spheroids suggesting cytoskeletal reorganization, while Panc1 cells
396 exhibited a decrease in cell-to-cell contacts. In addition, Panc1 CSCs were rounded and detached after
397 atorvastatin treatment.

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401

402 **DISCUSSION**

403 **Activated metabolic pathways in Panc1 CSCs**

404 Our previous study [9] showed higher secretion level of different proteins in Panc1 CSCs compared
405 with the parental cells, and hence suggested possible involvement of these proteins in the processes of
406 pancreatic cancer differentiation, invasion, and metastasis.

407 In the present study, with the aim of further deepening the understanding of pancreatic CSC
408 biology, we carried out a proteomic analysis of Panc1 and Panc1 CSC whole-cell extracts to
409 investigate the intracellular molecular mechanisms characterizing pancreatic CSCs. The data obtained
410 revealed the regulation of some key metabolic pathways in Panc1 CSCs (**Fig. 9**).

411 Our proteomic results indicate that glycolysis and gluconeogenesis (**Fig.1** and **Fig. 3**),
412 previously reported to be involved in the secretome of both Panc1 cells and Panc1 CSCs [9], are also
413 strongly represented by intracellular up-regulated proteins in Panc1 CSCs. In particular, data show that
414 fructose-bisphosphate aldolase A (ALDOA, +15.31), triosephosphate isomerase (TPI1, +13.25),
415 glyceraldehyde-3-phosphate dehydrogenase (GAPDH, +9.21), phosphoglycerate kinase 1 (PGK1,
416 +2.37), phosphoglucomutase-1 (PGM1, +18.29), phosphoglycerate mutase 1 (PGAM1, +12.26), alpha-
417 enolase (ENOA, +13.48), and pyruvate kinase isozymes M1/M2 (PKM, +18.76) are all more highly
418 expressed in Panc1 CSCs than in the parental cells. Furthermore, Panc1 CSCs also express an
419 increased level of both L-lactate dehydrogenase A and B chains (LDHA +10.12, LDHB +15.13),
420 which indicates an accentuation of the Warburg effect. According to these data, the upstream regulator
421 analysis performed *in silico* by IPA has identified, among other activated regulators, the transcription
422 factor HIF1A. Indeed, HIF1 α , which is typically up-regulated even in solid malignancies in normoxia,
423 modulates stem cell fate reprogramming through glycolytic shift and upregulation of PKM2 [15, 16].
424 Notably, it has also been demonstrated that HIF1A promotes pancreatic ductal adenocarcinoma
425 invasion and metastasis by activating transcription of the actin-bundling protein fascin [17]. In line
426 with this finding, we found that fascin is up-regulated 7.06 fold in Panc1 CSCs as compared to
427 parental cell line (see **Suppl. Table 4**).

428 In agreement with the increased expression of glycolytic enzymes, our metabolomic data show
429 that in CSCs the level of glucose-6 phosphate is enhanced, suggesting an increased activity of
430 exokinase in CSCs, despite its expression not differing between cell lines, as shown by the proteomic
431 data. The other glycolytic intermediates that were analysed were present at higher levels in CSC
432 compared to parental cells, though the 2-phosphoglycerate level was not significantly different,
433 confirming a more glycolytic metabolism for CSCs. In line with this observation and with the
434 proteomic data, the level of lactate is also significantly higher in CSCs than in parental cells.

435 The recent literature describes CSCs as being primarily glycolytic or preferentially relying on
436 oxidative phosphorylation depending on tumour-type and microenvironment [18]. However, it has
437 been shown that in glucose rich environments, proliferating CSCs primarily utilize aerobic glycolysis
438 for their bioenergetic needs, while in glucose (and oxygen) deprived conditions, CSCs shift to a
439 quiescent, slow cycling state relying on mitochondrial oxidative metabolism [19]. In particular, the
440 activation of the glycolytic programme favours stemness via various mechanisms [20, 21], including
441 enhanced antioxidative capacity, with pentose phosphate pathway (PPP) being the most relevant. Our
442 results indicate that several up-regulated proteins of Panc1 CSCs belong to “free radical scavenging”
443 among top biological functions (**Table 1**) and to PPP as a statically significant perturbed pathway (**Fig.**
444 **1** and **Fig. 3**). Among PPP enzymes, Panc1 CSCs show over-expression of glucose-6-phosphate 1-
445 dehydrogenase (G6PD, +5.61), 6-phosphogluconate dehydrogenase (PGD, +11.67), and transketolase
446 (TKT, +5.66). These results are also confirmed by the metabolomic analysis which highlights the
447 increase of PPP intermediates (**Fig. 9**). The PPP is a major glucose metabolic pathway required for
448 cellular demands of anabolism and antioxidant defence. Indeed, the main purpose of PPP is to
449 regenerate NADPH from NADP⁺ through an oxidation/reduction reaction. This reaction is coupled to
450 the formation of ribose 5-phosphate from glucose 6-phosphate, thus making PPP among the major
451 metabolic pathways involved in malignancies [22]. Our data show increase of metabolic intermediates
452 and byproducts especially of the oxidative phase of PPP (D-gluconic acid and D-ribose 5-phosphate).
453 The increase of oxidative phase intermediates might be explained through diversion to purine salvage

454 pathway (PSP), as observed in red blood cell [23]. Purine nucleotides may be synthesized in cells *de*
455 *novo* or reconstructed from already existing free purine bases through the salvage reactions
456 (reutilization) [23].

457 In Panc1 CSCs, in line with the up-regulation of enzymes involved in glycolysis and PPP, we
458 also observed an induction of enzymes involved in the pyruvate-malate cycle (citrate-pyruvate cycle)
459 (**Fig.1** and **Fig. 3**), i.e. the cytosolic ATP-citrate synthase (ACLY, +2.77), cytoplasmic malate
460 dehydrogenase (MDH1, +10.17) and cytosolic NADP-dependent malic enzyme (ME1, +12.19). This
461 pathway is responsible for citrate transport out of the mitochondria into the cytosol where it is cleaved
462 by ACLY to oxaloacetate and acetyl-CoA, which is available for fatty acid synthesis. On the other
463 hand, cytosolic oxaloacetate is hydrogenated by MDH1 to give NAD^+ and malate, which is then
464 oxidized by ME1 to pyruvate in a reaction which also provides NADPH mainly for fatty acid
465 synthesis. Thus, NADH generated in glycolysis is converted to NADPH for fatty acids synthesis, while
466 simultaneously regenerating the NAD^+ needed to continue glycolysis [24]. Recently, it has been
467 reported that PDAC cells rely on the pyruvate-malate cycle to increase the NADPH/NADP (+) ratio,
468 and that oxaloacetate is produced by a metabolic reprogramming mediated by the KRAS oncogene
469 [25]. Accordingly, we demonstrate that Panc1 cells, which are known to possess a KRAS mutation
470 [26], express enzymes of the pyruvate-malate cycle and that these enzymes are even more expressed in
471 Panc1 CSCs.

472 Consistently with the up-regulation of enzymes involved in glycolysis, PPP, and pyruvate-
473 malate cycle, we also found that Panc1 CSCs have a strong induction of two cytosolic enzymes
474 involved in lipid metabolism, the cytosolic acetylCoA acetyl transferase (ACAT2, +21.19 fold), which
475 synthesize acetoacetylCoA in the mevalonate pathway leading to cholesterol, and the fatty acid
476 synthase (FASN, +18.36 fold), which synthesize fatty acids.

477 Recently, in proliferating cells, it has been demonstrated that NADPH production, beyond the
478 important contribution of PPP and the malic enzyme (ME1), originates from the serine-driven one-
479 carbon metabolism, in which oxidation of methylene tetrahydrofolate to 10-formyl-tetrahydrofolate is

480 catalysed by the methylene tetrahydrofolate dehydrogenase (MTHFD) with the reduction of NADP to
481 NADPH [27]. Our data demonstrate for the first time that Panc1 CSCs are characterized by increased
482 levels of the cytosolic MTHFD1 (+3.19) compared to parental cells suggesting that stemness requires a
483 higher contribution of the folate pathway for NADPH homeostasis.

484

485 **Repressed pathways in Panc1 CSCs**

486 As a further confirmation of a switch to a glycolytic metabolism at the expense of the
487 mitochondrial oxidative metabolism, Panc1 CSCs also show a down-regulation of some key enzymes
488 of the Krebs cycle (**Supplemental Table 4**). In support of these results, the metabolomic data show
489 that the level of three Krebs cycle intermediates, i.e. succinate, fumarate, and malate, is significantly
490 decreased in CSCs in comparison to parental cells (**Fig. 5B**). Furthermore, glutamine level is decreased
491 in CSCs compared to P cells, suggesting either a less active glutamine-transport inside the cell or a
492 greater conversion of glutamine to glutamate. Indeed, glutamate level is enhanced in CSCs compared
493 to parental cells, suggesting a minor incorporation of glutamate in the mitochondrion and, together
494 with the other results, a less active Krebs cycle in CSCs in comparison to parental cells.

495 In Panc1 CSCs, proteins involved in spliceosome formation and non-homologous end joining
496 pathway appear to be down-regulated compared to Panc1 cells (**Fig.2** and **Fig.3**), in particular, several
497 hnRNPs (A1, A3, A/B, A2/B1, C1/C2, H3, K, L, M, U, Q), two splicing factors (3B subunit 2, 1
498 serine/arginine-rich), and the RNA-binding protein 25. The down-regulation of multiple types of
499 hnRNPs may represent a strategy of stem cells to keep their undifferentiated state. Indeed, it has been
500 demonstrated that hnRNPs play a key role in the stem cell differentiation process, as for example in the
501 case of embryonic stem cells differentiated into smooth muscle cell, or of multipotent hematopoietic
502 stem cells differentiated into the different types of blood cell, or of neural stem cell differentiated into
503 neuronal cells [28].

504 The non-homologous end-joining (NHEJ) pathway is one of the main mechanisms for repairing
505 breaks in double-stranded DNA. In Panc1 CSCs, representative proteins of this pathway (**Fig.2** and

506 **Fig.3**), i.e. poly [ADP-ribose] polymerase 1 (PARP1, -1.96), DNA-dependent protein kinase catalytic
507 subunit (PRKDC, -2.27), and X-ray repair cross-complementing proteins 5 and 6 (XRCC5 -1.89,
508 XRCC6 -1.92), are down-regulated. Interestingly, PARP1 is a pro-apoptotic enzyme [29], the
509 expression of which has been shown to be specifically reduced in stem cells with spherical
510 morphology as compared to monolayer cells [30]. Furthermore, XRCC5 and XRCC6 encode for Ku80
511 and Ku70 proteins, which form the Ku heterodimer that is involved in repairing double-strand breaks
512 in DNA for maintaining the integrity of genome function. Either Ku70 or Ku80 themselves have also
513 unique functions that are independent of the other Ku subunit [31], as for instance the tumour
514 suppressor activity of Ku70 [32]. In particular, it has been reported that colonic epithelial cells of mice
515 with Ku70 deficiency and p53^{R172P} mutation (Ku70^{-/-}PP) show higher rates of proliferation and
516 induction of β -catenin/Wnt pathway, which is important in promoting EMT. Notably, our previous
517 data demonstrated that Panc1 CSCs express higher levels of typical EMT markers compared to
518 parental cells [7].

519

520 **Functional role of fatty acid synthesis and mevalonate pathways in Panc1 CSC viability**

521 Among the most strongly induced proteins of Panc1 CSCs, we found of particular interest
522 FASN and ACAT2 that, as mentioned above, are involved in lipid metabolic pathways. Notably, high
523 expression level of FASN has been linked to a significantly poor prognosis of PDAC patients and it
524 has been shown to depend upon the induction of EGFR/ERK signalling [14]. Furthermore, FASN has
525 been shown to promote EMT in many cancers, including ovarian [33], breast [34] and colorectal [35]
526 cancer. Our results demonstrate that the overexpression of FASN in Panc1 CSCs is independent of the
527 presence of EGF and FGF in the medium, suggesting a constitutive activation of the EGFR/ERK
528 pathway.

529 Inhibition of FASN is known to selectively target cancer cells for apoptosis, mainly by
530 interfering with membrane function, inhibiting DNA replication and anti-apoptotic proteins, and
531 accumulating the malonyl-Co-A substrate [36]. Cerulenin is a natural FASN inhibitor that binds

specifically and irreversibly to the β -ketoacyl synthase domain of FASN, thus preventing the condensation reaction between the elongating fatty acid and successive malonyl residues. Interestingly, our results show that cerulenin inhibits Panc1 and Panc1 CSC proliferation, with a greater anticancer activity on stem cells (**Fig.6A**). This is a new observation that could open novel lines of investigation for the development of PDAC CSC target specific therapeutic strategies.

The other metabolic pathway that caught our attention was the mevalonate pathway, which is involved in cholesterol production, because of the strong up-regulation in Panc1 CSCs of ACAT2, one of its key enzymes. The mevalonate pathway affects cancer metastasis in several ways by influencing EMT, cytoskeleton remodelling, as well as cell motility and polarity (non-canonical Wnt/planar pathway). Statins are competitive inhibitors of this pathway by acting on the enzyme the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) and blocking the conversion of HMG-CoA to mevalonate. The statins occupy the catalytic site of HMGCR at the level of the binding site for HMG-CoA, thus blocking the access of this substrate to the active site. In the last two decades, the successful and widespread uses of statin drugs for hypercholesterolaemia have revealed their potential anticancer effects demonstrating that the use of statins is associated with reduced cancer-related mortality [37]. Inhibition of mevalonate pathway by statins has shown to have an antitumour effect against certain CSCs, but their effects have never been investigated on pancreatic CSCs [38]. Our data demonstrate that atorvastatin, a synthetic statin, inhibits cell growth of both Panc1 cells and Panc1 CSCs and that CSCs are considerably more sensitive than parental cells (**Fig.6B**), suggesting ACAT2 as a potential target for PDAC CSC specific therapy. In conclusion, the different cytotoxic effects by inhibiting fatty acid synthesis and mevalonate pathways on Panc1 and Panc1 CSCs suggest that the acute reduction of fatty acids or cholesterol production *per se* are not the major source of cell injury, but could target specific stemness characteristics.

In summary, the proteomic and metabolomic analysis on Panc1 cells and Panc1 CSCs highlighted the main metabolic routes used by pancreatic cancer stem cells to survive, proliferate and disseminate in distinct healthy tissues. In particular, these data indicate that the maintenance of high

558 fatty acid synthesis and mevalonate pathway levels could make critical contribution to survival of
559 pancreatic cancer stem cells. Although targeting tumour metabolism is still in the early days of
560 translation to patients, the improvement on knowledge of deranged metabolic pathways, in particular
561 in cancer stem cells, will accelerate the development of novel therapeutic strategies.

562

563

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656

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658

659 **Figure Legends**

660 **Figure 1**

661 Protein network of up-regulated proteins of Panc1 CSCs.

662 Schematic view of known and predicted protein interactions according to the STRING database (v.
663 10). Each node represents a protein, and each edge represents an interaction. Only interactions with the
664 highest confidence score (0.700) are shown. Interactions include physical and functional associations,
665 showing the evidence view. Thicker edges represent stronger associations.

666

667 **Figure 2**

668 Protein network of down-regulated proteins of Panc1 CSCs.

669 Schematic view of known and predicted protein interactions according to the STRING database (v.
670 10). Each node represents a protein, and each edge represents an interaction. Only interactions with the
671 highest confidence score (0.700) are shown. Interactions include physical and functional associations,
672 showing the evidence view. Thicker edges represent stronger associations.

673

674 **Figure 3**

675 IPA Canonical Pathways analysis.

676 The top 10 significantly altered canonical pathways associated with up-regulated (upper panel) and
677 down-regulated (lower panel) proteins of Panc1 CSCs. The y-axis indicates the statistical significance
678 calculated using the right-tailed Fisher exact test and the p value indicates the probability of
679 association of proteins with the canonical pathway by random chance alone. Thus, taller bars equate to
680 increased significance. The threshold line represents the default significance cut-off at $p = 0.05$. The
681 ratio is calculated as follows: the number of molecules found in a given pathway divided by total
682 number of molecules that constitute that specific canonical pathway.

683 The orange and blue coloured bars indicate predicted pathway activation, or predicted inhibition,
684 respectively (z-score). White bars are those with a z-score at or very close to 0. Grey bars indicate
685 pathways where no prediction can currently be made.

686

687 **Figure 4**

688 Western Blotting validation.

689 Differential expression of Panc1 CSC up-regulated (A) and down-regulated (B) proteins was verified
690 in two biological replicates (1 and 2). Proteins were resolved in 10-20% SDS-PAGE gels, transferred
691 onto PVDF membranes, and probed with specific antibodies against the indicated targets. Amido
692 Black staining was used as total loading control.

693

694 **Figure 5**

695 Metabolites analysis.

696 Absolute metabolomics quantification (arbitrary ion counts) of metabolites from glycolysis (A)
697 Pentose phosphate pathway (B) and Krebs cycle (C) in Panc1 parental cells (P; white histogram) and
698 Panc1 CSCs (black histogram). Values are presented as mean \pm SD. The data were analyzed using
699 Student's t-test: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ in Panc1 CSCs *versus* Panc1 P cells.

700

701 **Figure 6**

702 Anticancer effects of fatty acid and mevalonate pathways inhibition.

703 Cerulenin and atorvastatin effectively inhibited Panc1 CSCs viability. (A) Comparison of cerulenin
704 effects on Panc1 and Panc1 CSC cells viability. Cells were treated for 48 hours at doses ranging from
705 0 to 500 μ M. (B) Evaluation of atorvastatin effects on Panc1 and Panc1 CSC cells viability. Cells were
706 treated for 48 hours at doses ranging from 0 to 250 μ M. The data were analyzed using Student's t-test.

707 * $P, 0.05$.

708

709 **Figure 7**

710 Western Blotting analysis of Panc1 CSCs.

711 (A) FASN protein levels in Panc1 CSCs control (CTRL) and treated with cerulenin, and grown
712 without EGF and FGF (- EGF/FGF). (B) ACAT2 protein levels in Panc1 CSCs control (CTRL) and
713 treated atorvastatin. Proteins were resolved in 10-20% SDS-PAGE gels, transferred onto PVDF
714 membranes, and probed with specific antibodies against the indicated targets. Amido Black staining
715 was used as total loading control.

716

717 **Figure 8**

718 Cellular morphology after inhibition of fatty acid synthesis and mevalonate pathways.

719 Phase-contrast microscopy images of Panc1 and Panc1 CSCs after 24 hour of cerulenin (A) and
720 atorvastatin (B) treatments.

721

722 **Figure 9**

723 Overview of key metabolic pathways in Panc1 CSCs. The up- and down-regulated proteins (enzymes)
724 and metabolites identified in Panc1 CSCs compared to Panc1 P and mentioned into the discussion are
725 shown in bold red and green, respectively. Proteins and metabolites that were not identified in this
726 work are shown in gray. The inhibitors are shown in light blue.

727

728

729 **Table 1.** IPA-predicted top biological functions for up-regulated and down-regulated proteins of Panc1
730 CSCs.
731

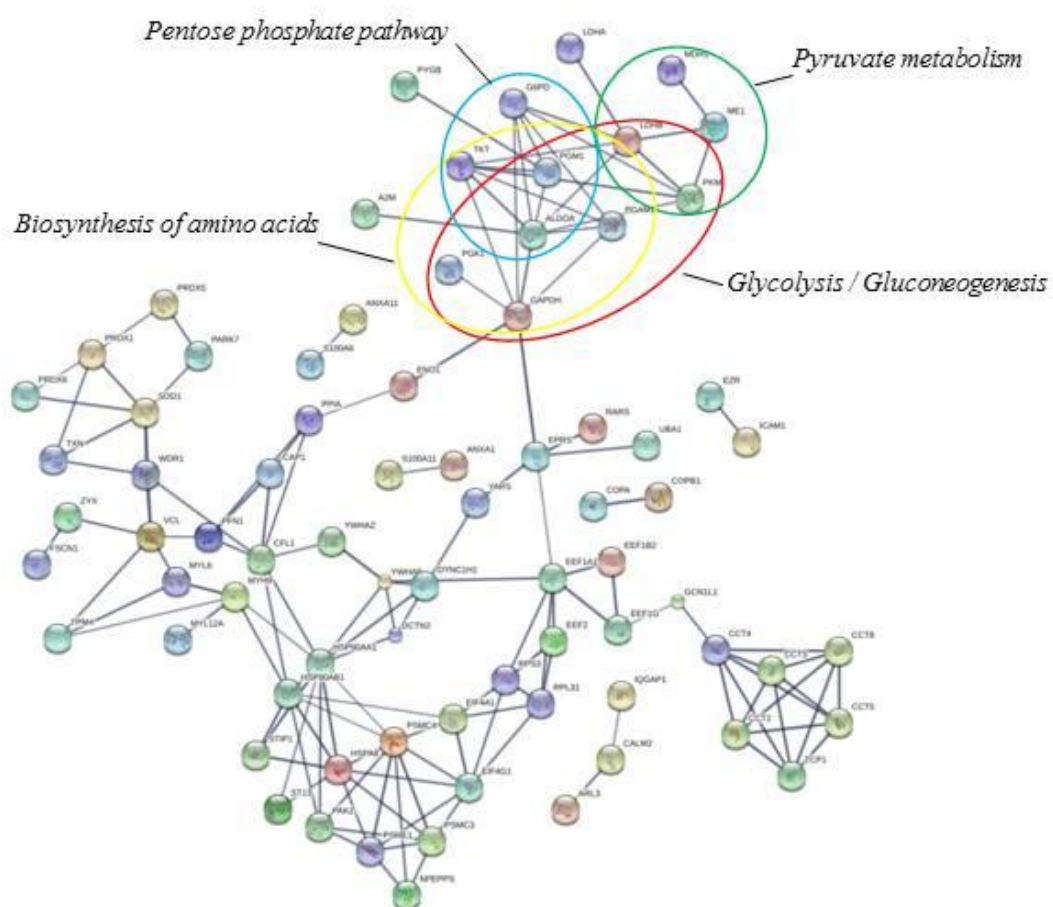
Top Biological Functions (Panc1 CSCs up-regulated proteins)	P values ^a (ranging from)	No. of molecules ^b
<i>Diseases and Disorders</i>		
Dermatological Diseases and Conditions	1.21E-02 - 1.05E-10	34
Neurological Disease	1.21E-02 - 9.23E-09	49
Skeletal and Muscular Disorders	1.16E-02 - 9.23E-09	50
Psychological Disorders	1.21E-02 - 9.54E-09	34
Cancer	1.25E-02 - 3.13E-08	102
<i>Molecular and Cellular Functions</i>		
Cellular Growth and Proliferation	1.21E-02 - 1.17E-15	58
Cell Death and Survival	1.21E-02 - 1.80E-11	52
Cellular Development	1.21E-02 - 8.62E-08	34
Cellular Movement	1.24E-02 - 1.22E-06	30
Free Radical Scavenging	1.08E-02 - 1.50E-06	12
<i>Physiological System Development and Function</i>		
Cell-mediated Immune Response	1.21E-02 - 3.47E-05	4
Hematological System Development and Function	1.21E-02 - 3.47E-05	14
Immune Cell Trafficking	1.21E-02 - 3.47E-05	10
Endocrine System Development and Function	1.21E-02 - 5.40E-04	4
Organismal Survival	7.10E-04 - 7.10E-04	9

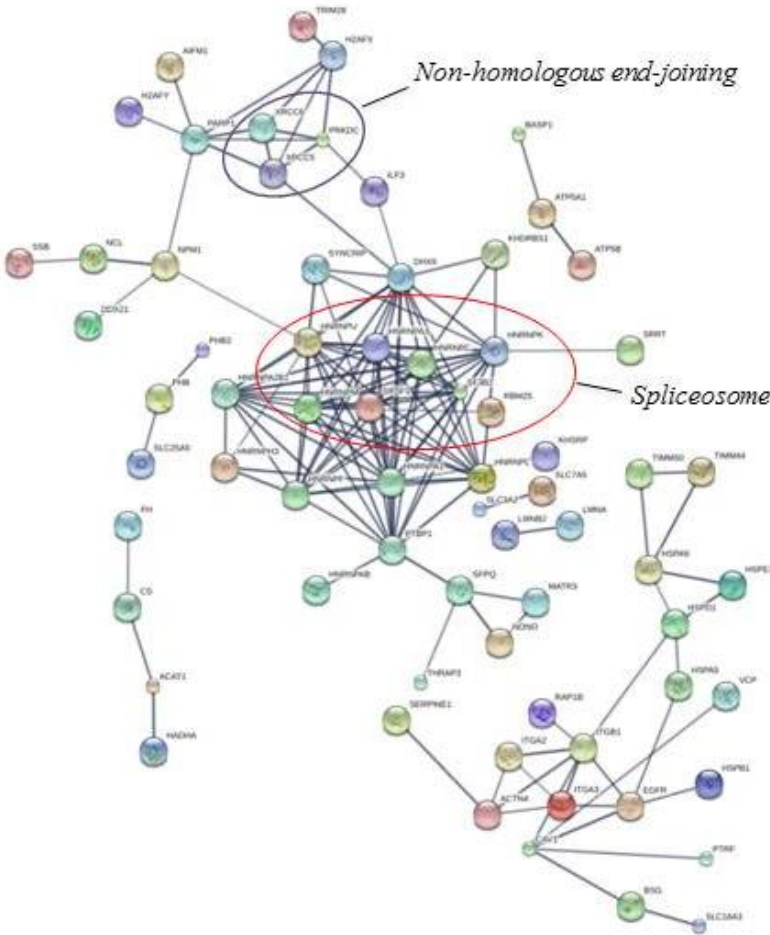
Top Biological Functions (Panc1 CSCs down-regulated proteins)	P values ^a (ranging from)	No. of molecules ^b
<i>Diseases and Disorders</i>		
Infectious Diseases	6.07E-03 - 2.85E-08	33
Dermatological Diseases and Conditions	6.07E-03 - 1.14E-07	22
Cancer	6.07E-03 - 2.15E-07	105
Organismal Injury and Abnormalities	6.07E-03 - 2.15E-07	105
Neurological Disease	6.07E-03 - 6.74E-07	42
<i>Molecular and Cellular Functions</i>		
Cellular Growth and Proliferation	6.07E-03 - 1.56E-18	64
RNA Post-Transcriptional Modification	6.07E-03 - 2.22E-17	23
Cell Death and Survival	6.07E-03 - 2.24E-15	54
Cellular Development	6.07E-03 - 8.12E-10	55
Cellular Response to Therapeutics	1.00E-03 - 1.48E-09	7
<i>Physiological System Development and Function</i>		
Skeletal and Muscular System Development and Function	1.09E-04 - 8.12E-10	12
Tissue Development	6.07E-03 - 8.12E-10	30
Cardiovascular System Development and Function	6.07E-03 - 3.70E-07	18
Organismal Development	6.07E-03 - 3.70E-07	21
Hair and Skin Development and Function	6.07E-03 - 2.03E-05	11

732 ^a Fisher's exact test was used to calculate a p value for each protein of the dataset identified in the biological function studied, indicating the probability
733 that each biological function assigned to the data set is assigned by chance; then we have a range of p values corresponding to all p values calculated for
734 all proteins of the dataset in the biological function. ^b The number of molecules of the differentially expressed protein dataset is shown.
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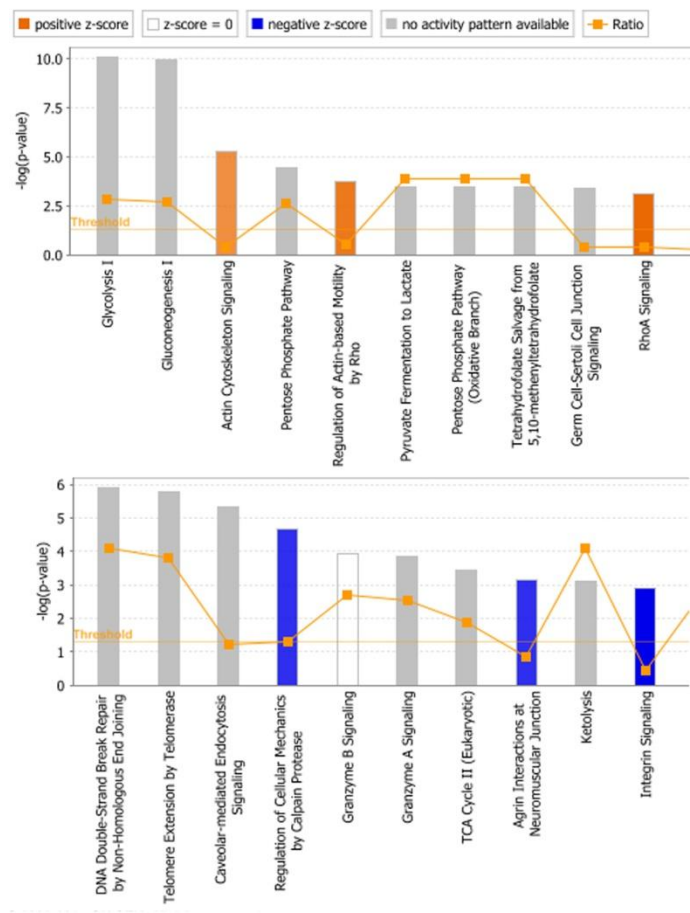
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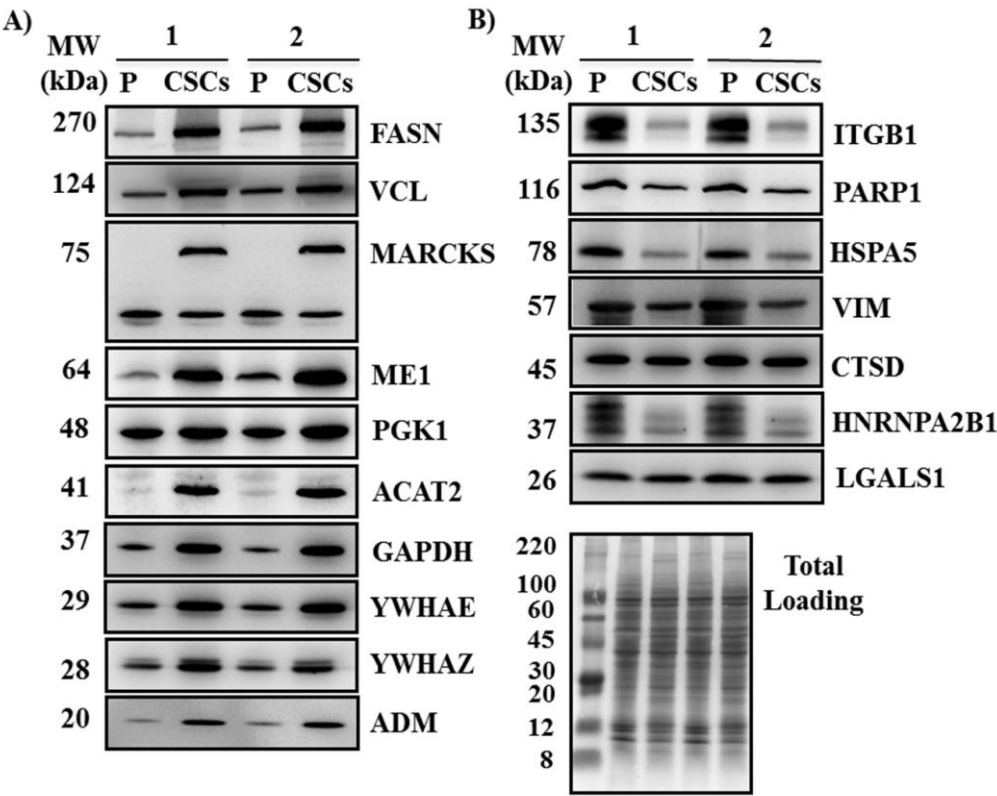
755 **Figure 3**

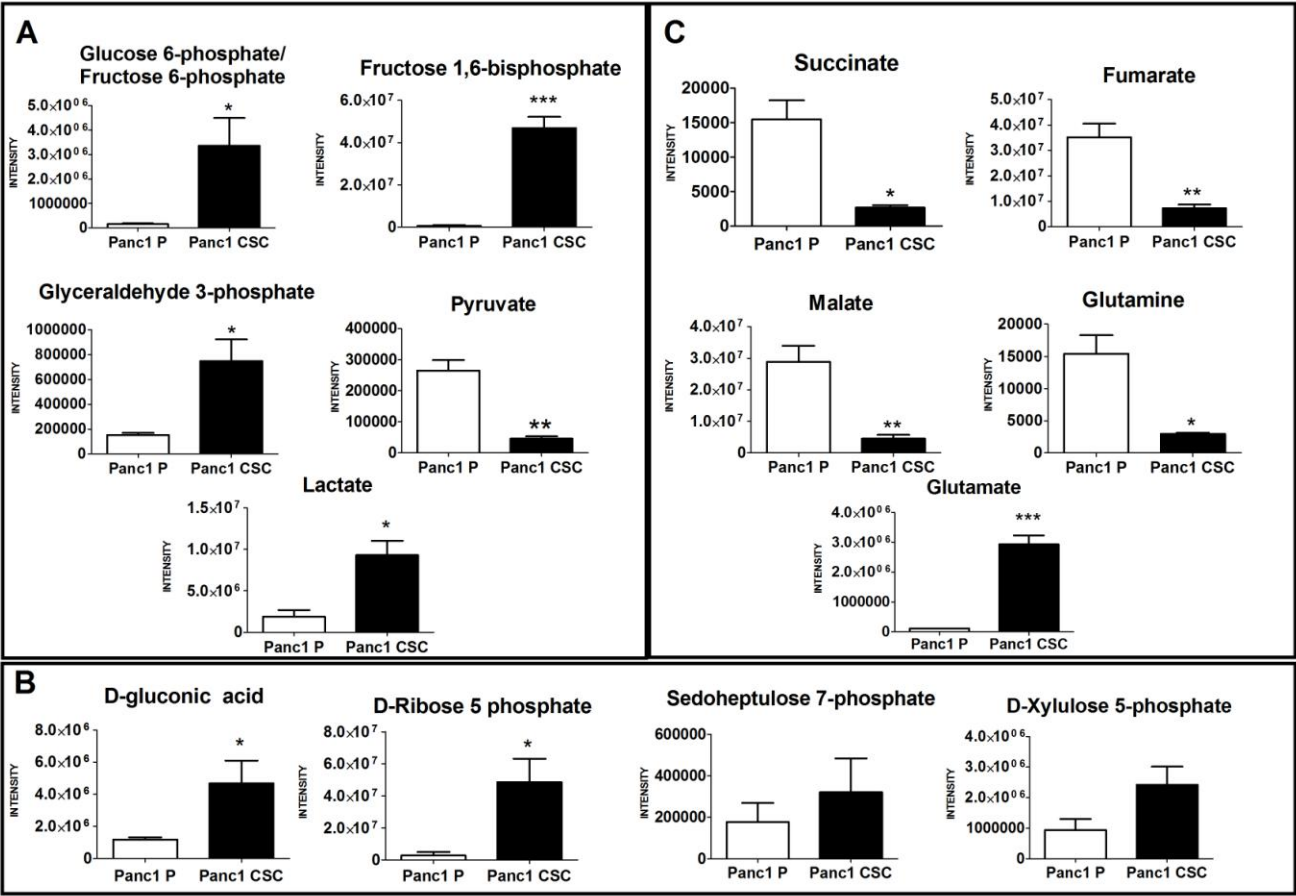


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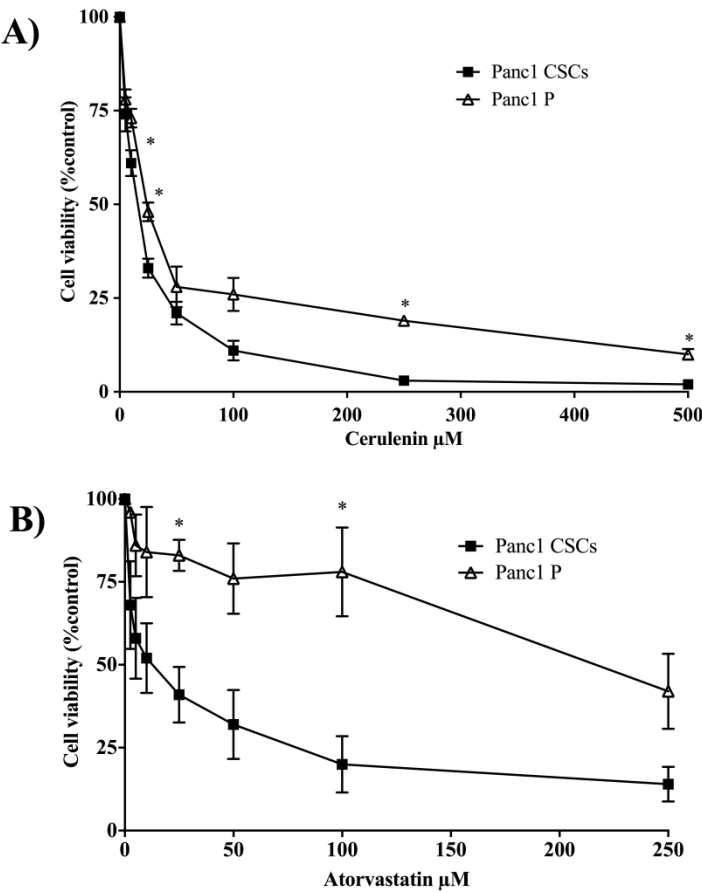
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758 **Figure 4**





778 **Figure 6**

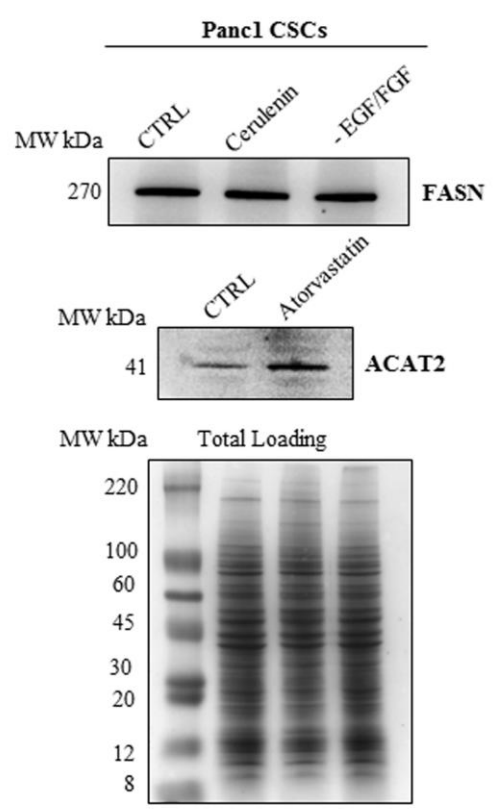


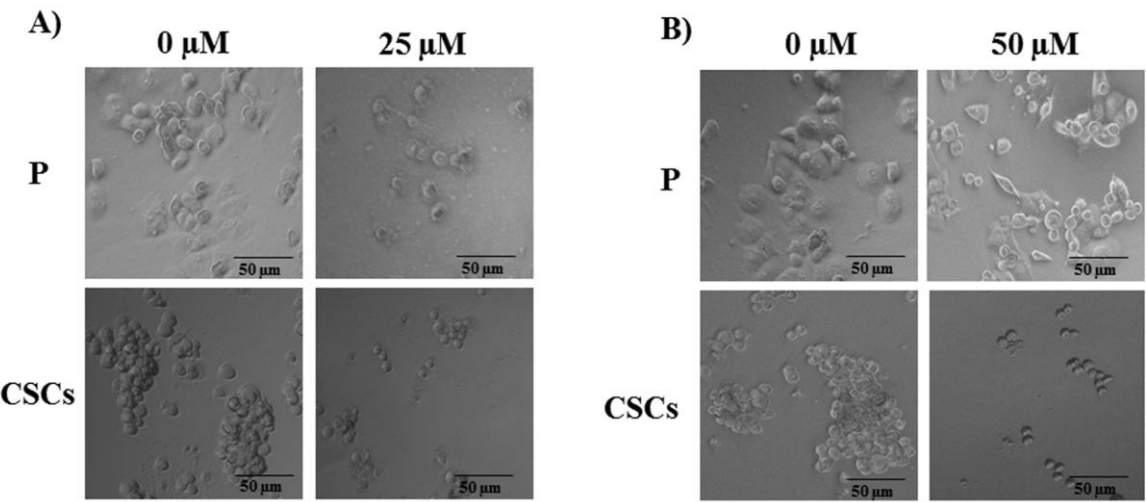
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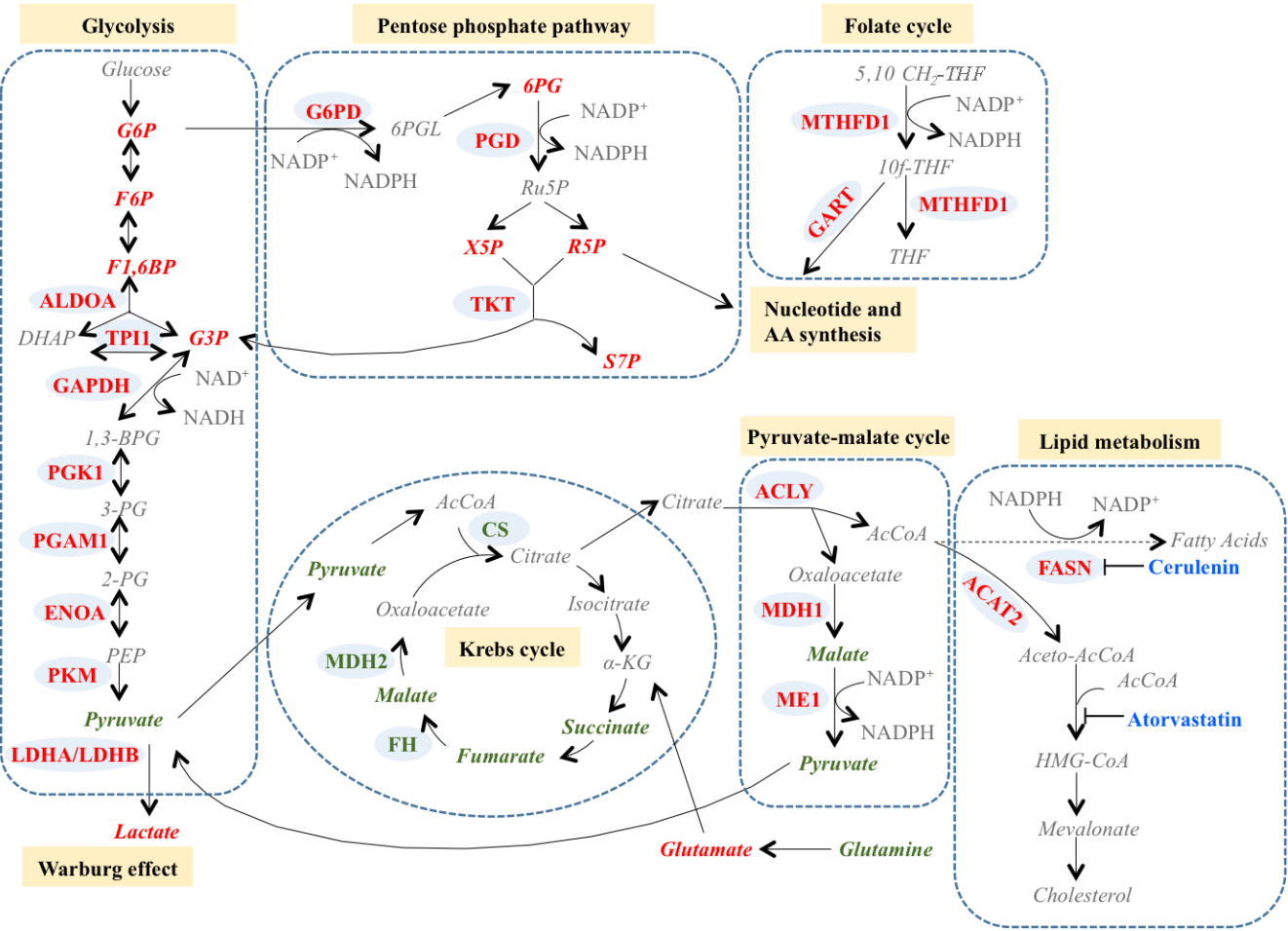
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787 **Figure 9**



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supplemental Figure 1 (networks alterations)

[Click here to download Supplementary material: supplemental Figure 1 \(networks alterations\).doc](#)

Supplemental Table 3 (n=608 identification via single peptide)
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Supplemental Table 4 (n=230 differentially expressed)

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Supplemental Table 5 (Canonical pathways enriched)

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Supplemental Table 6 (upstream regulators)

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***Conflict of Interest**

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